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Activation of c-Src in Cells Bearing v-Crk and Its Suppression by Csk

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The protein product of the CT10 virus, p47$^{gag\text{-}crk}$ (v-Crk), which contains Src homology region 2 (SH2) and 3 (SH3) domains but lacks a kinase domain, is believed to cause an increase in cellular protein tyrosine phosphorylation. A candidate tyrosine kinase, Csk (C-terminal Src kinase), has been implicated in c-Src Tyr-527 phosphorylation, which negatively regulates the protein tyrosine kinase of pp60$^{c\text{-}src}$ (c-Src). To investigate how c-Src kinase activity is regulated in vivo, we first looked at whether v-Crk can activate c-Src kinase. We found that cooverexpression of v-Crk and c-Src caused elevation of c-Src kinase activity, resulting in an increase of tyrosine phosphorylation of cellular proteins and morphological transformation of rat 3Y1 fibroblasts. v-Crk and c-Src complexes were not detected, although v-Crk bound to a variety of tyrosine-phosphorylated proteins in cells overexpressing v-Crk and c-Src. Overexpression of Csk in these transformed cells caused reversion to normal phenotypes and also reduced the level of c-Src kinase activity. However, Csk did not cause reversion of cells transformed by v-Src or c-Src527F, in which Tyr-527 was changed to Phe. These results strongly suggest that Csk acts on Tyr-527 of c-Src and suppresses c-Src kinase activity in vivo. Because Csk can suppress transformation by cooverexpression of v-Crk and c-Src, we suggest that v-Crk causes activation of c-Src in vivo by altering the phosphorylation state of Tyr-527.

An increasing number of proteins have been shown to contain Src homology region 2 (SH2) and/or 3 (SH3) domains (6, 23, 41). These domains may function in cellular regulation by mediating protein-protein interactions. Indeed, many studies have shown that the SH2 domains of viral Crk (the Gag–v-Crk fusion protein will be referred to hereafter as v-Crk in this article for convenience) (1, 27, 28, 31, 34), Src (1, 27, 28), Abl (33), Ras GTPase-activating protein (1, 34), the p85 subunit of phosphatidylinositol 3′ kinase (20, 44), phospholipase Cγ (1, 46), and the oncoprotein Vav (5, 26) can bind to a variety of tyrosine-phosphorylated proteins, including the epidermal growth factor receptor, platelet-derived growth factor receptor, colony-stimulating factor 1 receptor, and c-Src, v-Src, and their phosphorylated substrate proteins. The SH3 domain alone has also been identified in a variety of proteins, for some of which the role in membrane association or cytoskeletal association has been implicated (23).

The v-Crk protein is composed of only SH2 and SH3 domains and has no kinase activity, although cells expressing v-Crk contain elevations in cellular protein tyrosine phosphorylation (29–31), which suggests that v-Crk might act as a regulatory component of other cellular protein(s). It has been shown that the SH2 domain of v-Crk can bind to a broad range of tyrosine-phosphorylated proteins when they are incubated in vitro (27, 34) and that such bindings can protect the proteins from dephosphorylation by protein tyrosine phosphatases (3). Also, since cellular protein tyrosine kinase activities are coimmunoprecipitated with v-Crk, it is suggested that v-Crk might activate or deregulate some kinases by binding to the kinases (30). However, the total kinase activity coprecipitated with v-Crk was even less than that of endogenous c-Src in chicken embryo fibroblasts (31). The natures of these kinases are unknown, and the mechanism as to how v-Crk increases protein tyrosine phosphorylation and subsequent cellular transformation remains to be determined.

The kinase activity of c-Src is regulated by phosphorylation of Tyr-527, the major site of tyrosine phosphorylation in vivo (10, 17). Dephosphorylation of this residue causes a 10- to 20-fold increase in c-Src kinase activity in vitro (11, 12). Also, the dephosphorylated c-Src is believed to become phosphorylated on Tyr-416 by autophosphorylation activity of the c-Src kinase in vivo (4, 12, 15, 17). Autophosphorylation on Tyr-416 may positively modulate the activity of c-Src (21). Mutational analysis also showed the significance of Tyr-527 phosphorylation in regulating c-Src kinase activity in vivo (7, 22, 42). We and other groups have suggested that this phosphorylation is mainly mediated not by inter- or intraphosphorylation of c-Src kinase but by another kinase(s) (18, 24, 52). Recently, such a candidate protein tyrosine kinase, C-terminal Src kinase (Csk), has been purified from the membrane fraction of rat brains and Csk cDNA has been identified (37, 39). Chicken Csk cDNA has been also identified and a high degree of conservation between rat and chicken Csk cDNA sequences was shown (48). Csk specifically phosphorylated Tyr-527 of c-Src in vitro or in *Saccharomyces cerevisiae* cells by cDNA transfer (37–39). The decrease in c-Src kinase activity was demonstrated in vitro after c-Src phosphorylation by Csk (39). It has also been demonstrated that Csk can phosphorylate other members of the Src family of kinases like p56^{lyn} and p59^{fyn} at the carboxyl-terminal tyrosine residue corresponding to Tyr-527 of c-Src (38).

To analyze the mechanism of regulation of c-Src kinase activity in vivo and also to obtain information about the mechanism of v-Crk transformation, we looked at whether c-Src can be activated by v-Crk in vivo. Here we report that the kinase activity of overexpressed c-Src is elevated above

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the basal level in cells bearing v-Crk and is restored to the basal level by Csk overexpression. Interactions among v-Crk, c-Src, and Csk at the molecular level will be discussed.

MATERIALS AND METHODS

Cells, plasmids, and antibodies. Cos 7 cells and 3Y1 cells were maintained in Dulbecco's modified Eagle's medium containing 10 and 5% fetal calf serum, respectively. The mammalian expression vector pBaBePuroc-*src* and pcDNAI *csk* (sense and antisense orientations in relation to the promoter) have been described previously (48). pBaBe Purov-*src* was made by ligating the 2.3-kb *Xmn*I-*Nru*I v-*src* fragment from pSR-XD2 (13) into the *Sna*BI site of pBaBePuro vector (36) (kind gift of H. Land, National Cancer Institute, Frederick, Md.) in the sense orientation relative to the promoter. pMAM*csk* was made by ligating the 2.4-kb chicken *csk* cDNA (48) into the *Sma*I site of the pMAM vector (Clontech Laboratory Inc., Palo Alto, Calif.) either in the sense or antisense orientation relative to the promoter, after blunt ending both the insert cDNA fragment and the vector fragment with T4 polymerase. pMexNeov-*crk* was made by ligating the 1.8-kb *Alw*NI *gag* v-*crk* fragment from CT10 virus DNA into the *Kpn*I site of the pMexNeo vector in the sense orientation relative to the promoter (32). Cos 7 cells were transfected with plasmid DNA by the DEAE-dextran method as described previously (48). 3Y1 cells were transfected with linearized plasmid DNA by the calcium phosphate method (14), and stable transformants were selected with 0.5 mg of G418 per ml for pMexNeov-*crk*, 1.5 μg of puromycin per ml for pBabePuroc-*src* and pBabePurov-*src*, and 0.2 mg of hygromycin per ml for pBaBeHygro. The enzymes used to linearize plasmid DNA were *Not*I for pBaBe, *Nhe*I for pcDNAI, *Nde*I for pMAM, and *Sca*I for pMexNeo. For stable transfection of *csk* cDNA, pBaBe Hygro (36) (kind gift of H. Land, National Cancer Institute) plasmid DNA was cotransfected as a selection marker after linearization with *Not*I.

Monoclonal antibody (MAb) 2-17 (SCRF 35.4), which recognizes amino acids 2 to 17 of Src, was purchased from Quality Biotech (Camden, N.J.). MAb 327 (25) specific for Src was provided by J. Brugge (University of Pennsylvania), and MAb 3C2 (43) specific for viral Gag protein was provided by D. Boettiger (University of Pennsylvania). Polyclonal anti-Crk (30), anti-Csk (37), and antiphosphotyrosine (29) antibodies were described previously.

Immunoblotting. For Western blot (immunoblot) analysis of cellular proteins, cells were solubilized with Laemmli sodium dodecyl sulfate (SDS) sample buffer containing 5 mM EDTA and 1 mM Na_3 VO_4 and boiled for 5 min. Western blot analysis was performed as described previously (49). After the filters were incubated and washed, antibodies retained on the filter (Immobilon P; Millipore) were detected with ^{125}I-labeled protein A (ICN) for polyclonal antiphosphotyrosine antibodies and polyclonal anti-Crk and polyclonal anti-Csk antibodies, or with ^{125}I-labeled anti-mouse immunoglobulin G polyclonal antibody (Amersham) for MAb 327.

In vitro kinase assay. Harvested cells were lysed in RIPA buffer (1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 20 mM Tris-HCl [pH 7.4], 150 mM NaCl, 5 mM EDTA, 1 mM Na_3VO_4, 10 μM Na_2MoO_4, 1 mM phenylmethylsulfonyl fluoride, 20 μg of aprotinin per ml, 20 μg of leupeptin per ml, 10 mM NaF), and c-Src was immunoprecipitated with MAb 327 coupled with protein A-Sepharose. The purified c-Src was incubated in 50 μl of a solution containing 0.1% Triton X-100, 20 mM Tris-HCl (pH 7.2), 0.2 mM Na_3VO_4, 5 mM $MgCl_2$, and [γ-^{32}P]ATP (10 μCi; 3,000 Ci mmol^{-1}) with 2 μg of acid-denatured rabbit muscle enolase at 30°C for 10 min and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE).

Cyanogen bromide cleavage of Src proteins. Cyanogen bromide cleavage of the c-Src protein was done as described previously (18), with some modifications. Briefly, c-Src protein labeled with ^{32}P in vivo by incubating cells with 1 mCi of carrier-free $^{32}P_i$ (Amersham) per ml for 4 h in phosphate-free Dulbecco's modified Eagle medium was purified by immunoprecipitation with MAb 327 in RIPA buffer and was further purified by SDS-PAGE. After electrophoresis, the 60-kDa band was excised, washed in 10% methanol, and lyophilized. The piece of gel containing the band was incubated with 70% formic acid containing 50 mg of CNBr per ml for 2 h at room temperature, washed with water, and lyophilized. The proteins were then extracted in a solution containing 50 mM NH_4HCO_3, 0.1% SDS, and 1% β-mercaptoethanol, lyophilized again, boiled in Laemmli SDS sample buffer, and run on SDS–18.75% polyacrylamide gels containing 6 M urea.

Immunoprecipitation. To analyze v-Crk or c-Src complexes, the cells were solubilized in a buffer containing 1% Nonidet P-40, 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 20 μg of aprotinin per ml, 1 mM Na_3VO_4, 10 μM Na_2MoO_4, and 20 μg of leupeptin per ml. After clarification by centrifugation, v-Crk was precipitated with anti-Gag MAb 3C2, and c-Src was precipitated with MAb 2-17. Immunoprecipitates were analyzed by SDS-PAGE followed by Western blotting with polyclonal antiphosphotyrosine antibodies.

RESULTS

Protein tyrosine phosphorylation in Cos 7 cells by v-*crk* and c-*src* cotransfection. To determine whether v-Crk and c-Src cooperate or whether c-Src kinase is activated by v-Crk, both cDNAs were cotransfected into Cos 7 cells. Cell lysates were prepared 72 h after transfection, and protein tyrosine phosphorylation was analyzed by immunoblotting with polyclonal antiphosphotyrosine antibodies (Fig. 1). v-*crk* cDNA transfection caused clear induction of tyrosine phosphorylation on a protein of approximately 130 kDa, as detected in CT10 virus-infected chicken embryo fibroblast cells (29, 30) or 3Y1 cells expressing v-Crk by cDNA transfection (28). v-*src* cDNA transfection caused the appearance of many tyrosine-phosphorylated bands, whereas c-*src* cDNA transfection led to only one dominant tyrosine-phosphorylated protein (60 kDa) corresponding to c-Src itself. With v-*crk* and c-*src* cotransfection, a protein of approximately 70 kDa was clearly detected to be tyrosine phosphorylated in addition to those seen in either v-*crk* or c-*src* transfectants. Since the same-size protein was also strongly phosphorylated in v-*src*-transfected cells, this protein might have been phosphorylated by c-Src kinase activity.

c-*src* cDNA transfection causes morphological transformation of 3Y1 cells bearing v-*crk*. To further analyze the molecular basis for the increased protein tyrosine phosphorylation observed in the Cos 7 transient expression system, rat 3Y1 fibroblast cells were transfected with v-*crk* or c-*src* cDNA or both cDNAs and stable transfectants were selected by culturing in the presence of an appropriate drug. The cells in each colony were classified into four morphological types (types I to IV, as shown in Fig. 2), and the degree of

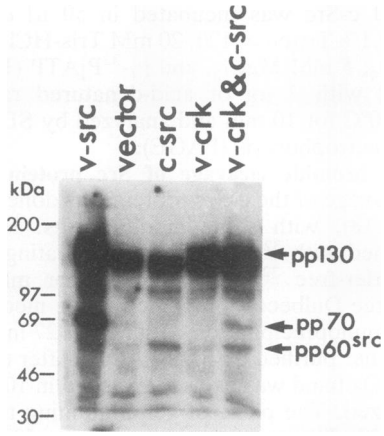

FIG. 1. Protein tyrosine phosphorylation in Cos 7 cells transfected with v-*crk* and/or c-*src* plasmid DNAs. Cos 7 cells (10^6) were transfected with 10 μg of plasmid DNA. Cell extracts were prepared 72 h after transfection and analyzed by immunoblotting with polyclonal antiphosphotyrosine antibodies as described in Materials and Methods. For cotransfection of different plasmid DNAs, 5 μg of each DNA was used.

morphological changes was expressed by the percentage of colonies in the morphological types. Although the v-*crk* oncogene causes transformation in chicken embryo fibroblast cells (29), only marginal morphological transformation was detected in 3Y1 cells (Table 1). c-*src* cDNA transfection did not cause any significant transformation, as expected (16, 40, 50) (Table 1). However, detectable morphological

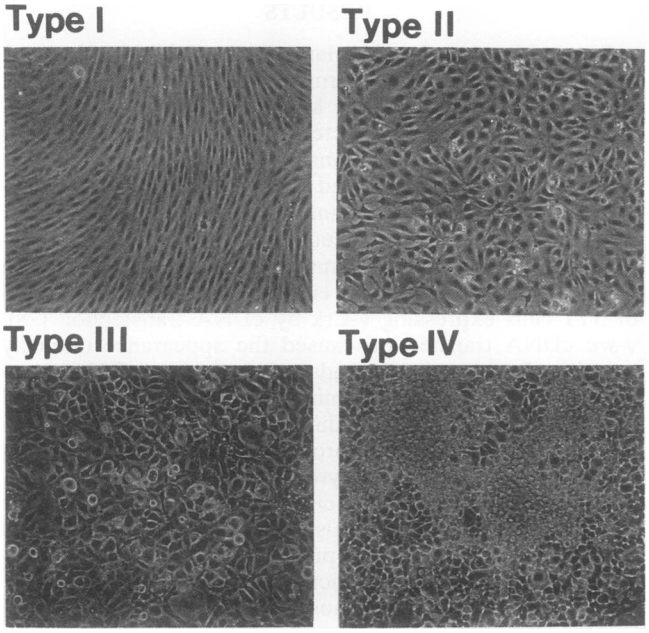

FIG. 2. Transformation of 3Y1 cells by v-*crk* and c-*src* cDNAs. Cells (2×10^5) in a 10-cm-diameter plate were transfected with 2 μg of linearized plasmid DNA and 38 μg of salmon sperm DNA as the carrier DNA, and stable transformants were selected as described in Materials and Methods. Representative 3Y1 cells after v-*crk* or v-*crk* and c-*src* cDNA transfection that exhibit the different morphological types (types I to IV) are displayed (see footnote *c* of Table 1 for details). The photographs were taken 11 days after selection.

TABLE 1. Transforming activities of c-*src* in 3Y1 cells expressing v-*crk*

Cell[a] and expt no.	DNA[b]	% of colonies with the following morphology after transfection[c]:			
		Type I	Type II	Type III	Type IV
3Y1	Vector	95.7	4.3	ND[d]	ND
	c-*src*	90.3	9.7	ND	ND
	v-*crk*	81.5	16.3	2.2	ND
	v-*src*	44.2	8.4	13.6	33.8
3Y1 (v-*crk*) Mixed	Vector	89.0	11.0	ND	ND
	c-*src*	58.0	15.1	21.2	5.7
Clone 1	Vector	94.7	5.3	ND	ND
	c-*src*	91.2	8.8	ND	ND
	v-*src*	36.1	16.2	15.3	32.4
Clone 2 Expt 1	Vector	93.2	6.8	ND	ND
	c-*src*	58.1	19.6	22.3	ND
Expt 2	Vector	93.2	6.8	ND	ND
	c-*src*	58.3	20.0	21.7	ND
	v-*src*	8.4	12.6	18.2	60.8

[a] Mixed 3Y1(v-*crk*) cells were made by pMexNeov-*crk* DNA transfection and selection with G418 for 2 weeks as described in Materials and Methods, and G418-resistant cells were pooled and used for further DNA transfections. 3Y1(v-*crk*) clone 1 and 3Y1(v-*crk*) clone 2 were isolated independently from type I transfectants of v-*crk*.

[b] pMexNeov-*crk*, pBaBePuroc-*src*, and pBaBePurov-*src* were used for v-*crk*, c-*src*, and v-*src* cDNAs, respectively. pBaBePuro was used for vector as a negative control.

[c] At 11 days after selection of cells either with puromycin for c-*src* and v-*src* or with G418 for v-*crk*, the numbers of cell colonies in each morphological type were scored. More than 200 colonies were counted in each transfection. Type I represents normal morphology. Types II to IV classify transformed phenotypes according to the degree of morphological alteration or saturation density of the cell colonies. Representative cells for each type are shown in Fig. 2.

[d] ND, not detectable.

transformation was observed when c-*src* was transfected into a pool of cells bearing v-*crk*, mixed 3Y1(v-*crk*) cells (Table 1). 3Y1 cell clones that expressed v-Crk but remained morphologically untransformed (type I [Fig. 2]) were then isolated and transfected with c-*src* cDNA. As shown in Table 1, c-*src* cDNA transfection caused morphological transformation in 3Y1(v-*crk*) clone 2 cells. Another cell clone, 3Y1(v-*crk*) clone 1, which expressed v-Crk at a level about 5 times lower (data not shown) than that of clone 2, showed only marginal transformation by c-*src* transfection (Table 1). Note that this cell clone can be transformed by v-*src* cDNA with an efficiency similar to that observed with 3Y1 cells. Those v-*crk*–c-*src*-transformed cells (type III [Fig. 2]) had five- or sixfold-higher densities at saturation than 3Y1(v-*crk*) clone 2 cells or normal 3Y1 cells did (data not shown).

Elevated protein tyrosine phosphorylation and c-Src kinase activity in 3Y1 cells overexpressing v-Crk and c-Src. Highly increased levels of tyrosine phosphorylation were found in several proteins in v-*crk*–c-*src*-transformed cells, whereas only p130 was phosphorylated in the v-*crk* cell line [3Y1(v-*crk*) clone 2] (Fig. 3). Interestingly, although the phosphorylation pattern in v-*crk*-c-*src* cells was somewhat similar to that in v-*src* cells, there were some differences in the relative intensities of phosphorylation (Fig. 3): for example, phosphorylation of p36, calpactin I heavy chain, was strong in

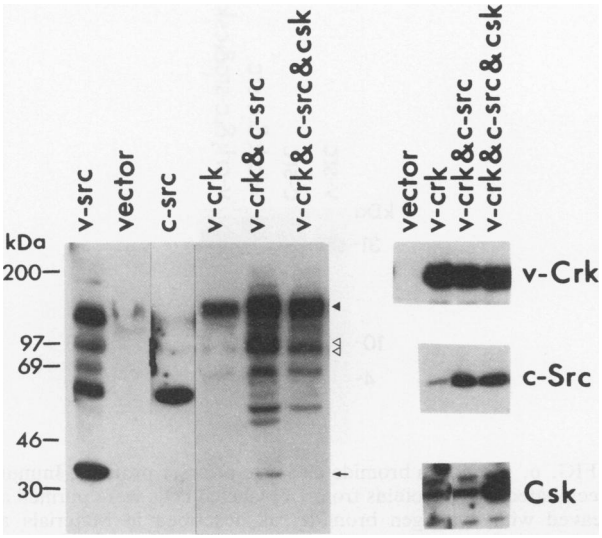

FIG. 3. Levels of cellular protein tyrosine phosphorylation and cDNA products in 3Y1 cells expressing v-Crk, c-Src, and Csk. Total cellular proteins (50 μg) were separated on a SDS-polyacrylamide gel, transferred to an Immobilon P filter, and probed with polyclonal antiphosphotyrosine antibody (left panel) as described in Materials and Methods. v-Crk, c-Src, and Csk were detected by using polyclonal anti-v-Crk antibodies, MAb 327, and polyclonal anti-Csk antibodies, respectively (right panels). For cells overexpressing c-Src, a longer exposure was used to show that c-Src expression causes no increase in cellular tyrosine phosphorylation. Molecular mass markers (Amersham) are shown to the left. p130 (◀), pp95 and pp90 (◁), and p36 or calpactin (←) are indicated (see text). The cell clones used were 3Y1(v-*crk*) clone 2 (Table 1) (v-crk lanes) and 3Y1 (v-*crk*-c-*src*) clone 21 (Table 2) (v-crk&c-src lanes) and its *csk*-reverted cell clone (v-crk&c-src&csk lanes) [Rev(II) (Fig. 5); Table 2]. v-src, c-src, and vector lanes represent 3Y1 cells expressing v-Src (type IV [Fig. 2]), c-Src (type I [Fig. 2]), and pBaBePuro, respectively, by cDNA transfection.

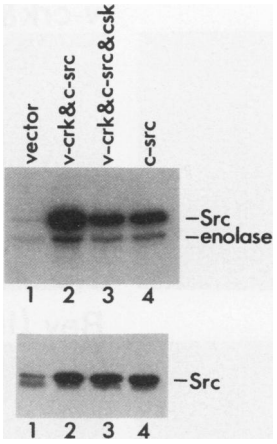

FIG. 4. In vitro kinase assay of c-Src. Cell lysates containing equal amounts of c-Src (except control cell lysate) were immunoprecipitated with MAb 327. Each immunoprecipitate was divided into two portions; one portion was incubated with acid-denatured enolase and [γ-^{32}P]ATP and subjected to SDS-PAGE (upper panel), as described in Materials and Methods. The other portion was immunoblotted and probed with MAb 327 to measure c-Src levels (lower panel). The same cell clones were used as described in the legend to Fig. 3. The relative phosphorylations quantitated with an image scanner (PhosphorImager 400E; Molecular Dynamics) were 0.06 (vector), 4.2 (v-*crk* c-*src* cells), 1.0 (v-*crk* c-*src csk* cells), and 0.88 (c-*src* cells) for c-Src and 0.32 (vector), 2.9 (v-*crk* c-*src* cells), 1.0 (v-*crk* c-*src csk* cells), and 0.87 (c-*src* cells) for enolase (upper panel). The relative amounts of c-Src proteins were 0.08 (vector), 1.2 (v-*crk* c-*src* cells), 1.0 (v-*crk* c-*src csk* cells), and 0.97 (c-*src* cells) (lower panel).

v-*src* cells, whereas its phosphorylation was negligible in v-*crk*-c-*src* cells. In addition, two bands of approximately 90 kDa (95 and 90 kDa) were detected in v-*crk*-c-*src* cells, whereas only one band was seen around 90 kDa in v-*src* cells. Bands of 50 to 55 kDa were detected only in v-*crk*-c-*src* cells. Overexpression of c-Src did not cause any increase in tyrosine phosphorylation of cellular proteins (Fig. 3). In vitro kinase assays revealed that, in these v-*crk*-c-*src* cells, c-Src showed a fourfold increase in autophosphorylation activity and a threefold increase in phosphorylation activity with enolase as the substrate (Fig. 4).

Csk overexpression can cause reversion of the morphological transformation of v-*crk*-c-*src* cells. In unsynchronized cell populations, c-Src has been shown to be phosphorylated at several residues, including Ser-12, Ser-17, and Tyr-527 (2, 8, 35, 51). Also, mitosis-specific hyperphosphorylations of c-Src at its amino-terminal region on amino acids Thr-34, Thr-46, and Ser-72 have been demonstrated (2, 8, 35, 51). Although these phosphorylations could contribute to the regulation of c-Src kinase activity in vivo, only Tyr-527 phosphorylation has been shown to significantly affect kinase activity when measured in vitro (9–11, 13, 17, 35, 51). Since elevated kinase activity was detected in vitro with c-Src from v-*crk*–c-*src*-transformed cells (Fig. 4), we speculated that this increased activity may be due to the alteration of Tyr-527 phosphorylation in vivo. Therefore, we examined the effects of Csk overexpression in these v-*crk*-c-*src* cells.

As shown in Table 2 and Fig. 5, after *csk* cDNA transfection, a significant fraction of the cells were flat. With pMAM*csk* transfection, in which an inducible mouse mammary tumor virus promoter was used, significant reversion was not

TABLE 2. Morphological reversion of 3Y1 (v-*crk* c-*src*) cells by *csk* cDNA transfection

3Y1(v-*crk* c-*src*) cell[a]	DNA[b]	% of colonies with the following morphology after transfection[c]		
		Nonreverted	Rev(I)	Rev(II)
Clone M1	pcDNAI*csk*	28.8	41.6	29.6
	pcDNAI*csk*(r)	91.4	6.9	1.7
Clone 21	pcDNAI*csk*	55.8	34.6	9.6
	pcDNA(I)*csk*(r)	90.6	6.7	2.7
Clone 25	pcDNAI*csk*	91.2	4.8	4.0
	pcDNAI*csk*(r)	88.6	7.0	4.4

[a] 3Y1(v-*crk* c-*src*) clone M1 and clone 21 were isolated from mixed 3Y1(v-*crk*) or 3Y1(v-*crk*) clone 2 cells, respectively, after transfection with pBaBePuroc-*src* DNA. Both cells showed type III morphology, as shown in Fig. 2. 3Y1(v-*crk* v-*src*) clone 25 is derived from 3Y1(v-*crk*) clone 2 cells transfected with pBaBePurov-*src*, which exhibit type III morphology, as shown in Fig. 2.

[b] Chicken *csk* cDNA in the pcDNAI expression vectors was cotransfected with pBaBeHygro DNA after being linearized as described in the legend to Fig. 5 and in Materials and Methods. DNA marked (r) contains cDNA fragments in the inverse orientation in relation the promoter.

[c] After 11 days of selection with hygromycin, the number of cell colonies was scored as described in footnote *c* of Table 1. Flat revertants are classified into two classes according to their morphology or saturation density. Rev(II) cells are flatter than Rev(I) cells; photographs are shown in Fig. 5.

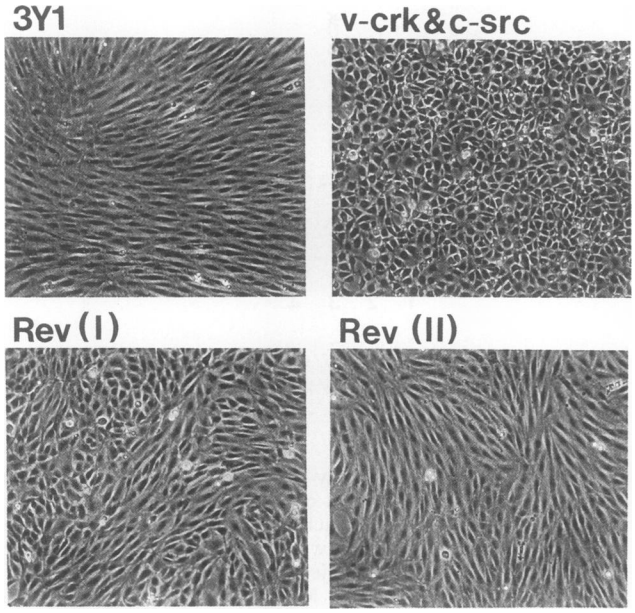

FIG. 5. Morphological reversion of v-*crk*–c-*src*-transformed cells by overexpression of Csk. Ten micrograms of linearized *csk* plasmid DNA was cotransfected with 0.5 μg of pBaBeHygro DNA in the presence of 30 μg of carrier DNA as described in the legend to Fig. 2. Rev(I) and Rev(II) cells were observed after *csk* cDNA transfection into v-*crk* c-*src* cells, which corresponded to type III in Fig. 2. (see footnote *c* of Table 2 for details). Photographs were taken 11 days after selection.

observed if the promoter was not induced. Of 12 clones isolated from pMAM*csk* transfectants, 4 were flat, like the Rev(II) cells in Fig. 5, when exogenous Csk protein was induced by the addition of dexamethasone (data not shown). In v-*crk* v-*src* cells, *csk* transfection did not cause significant reversion (Table 2). Moreover, Csk overexpression did not revert 3Y1 cells transformed by c-*src*527F, in which the codon for Tyr-527 was changed to Phe, causing constitutive activation of c-Src (22; also data not shown). The overexpression of Csk or the cooverexpression of v-Crk and Csk or of Csk and c-Src does not cause morphological transformation in 3Y1 cells, and changes in cellular protein tyrosine phosphorylation were not detected in Csk-overexpressing cells (data not shown). *csk*-reverted cell clones that were flat, such as the Rev(II) cells in Fig. 5, contained about threefold fewer cells at saturation density than v-*crk*–c-*src*-transformed cells (data not shown). That is, the cell density of these reverted cells was 1.5- to 2-fold higher than normal 3Y1 cells or 3Y1(v-*crk*) clone 2 cells at saturation.

Csk overexpression restored c-Src kinase activity to original levels and reduced protein tyrosine phosphorylation. In vitro c-Src kinase activity from these *csk*-induced revertants was reduced to a level similar to the basal level detected in c-Src-overexpressing cells (Fig. 4). Moreover, these cells showed somewhat lower levels of protein tyrosine phosphorylation than those of v-*crk* c-*src* cells (Fig. 3). v-Crk-induced phosphorylation of p130 remained unchanged. However, suppression of phosphorylation was most evident when phosphorylation of the 90- and 95-kDa bands were compared: although 95- and 90-kDa proteins were equally phosphorylated in v-*crk* c-*src* cells, phosphorylation of only 95-kDa protein decreased to an undetectable level in the reverted cells (Fig. 3).

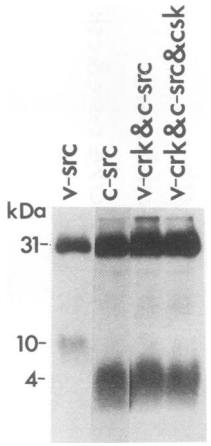

FIG. 6. Cyanogen bromide cleavage of c-Src proteins. Immunoprecipitated c-Src proteins from ^{32}Pi-labeled cells were purified and cleaved with cyanogen bromide, as described in Materials and Methods. v-Src proteins labeled in vivo were used as a control. The 10-kDa band contains Tyr-416, and the 4-kDa band contains Tyr-527. The same cell clones were used as described in the legend to Fig. 3.

c-Src is dominantly phosphorylated at Tyr-527 in both v-*crk*–c-*src*-transformed cells and *csk*-reverted cells. Cyanogen bromide cleavage analysis revealed that the c-Src protein in v-*crk* c-*src* cells was phosphorylated at Tyr-527 in vivo to a level similar to that detected in c-*src* cells (Fig. 6). The same result was true for c-Src protein in the *csk*-reverted cells (Fig. 6).

v-Crk binds to a variety of tyrosine-phosphorylated proteins, but not to c-Src, in v-*crk*–c-*src*-transformed cells. The v-Crk SH2 domain has been shown to bind to a variety of tyrosine-phosphorylated proteins (27, 28, 34). As shown in Fig. 7, almost all tyrosine-phosphorylated proteins detected in v-*crk* c-*src* cells coimmunoprecipitated with v-Crk from these cells. However, c-Src was not detected in this v-Crk immunoprecipitation prepared from v-*crk* c-*src* cells, by immunoblotting with MAb 327 (data not shown). Semiquantitative analysis revealed that we could detect c-Src protein in a v-Crk immunoprecipitate if more than about 1% of c-Src protein formed complexes with v-Crk in v-*crk* c-*src* cells (data not shown). Moreover, c-Src in v-*crk* c-*src* cells did not form such complexes with other tyrosine-phosphorylated proteins (Fig. 7), indicating that the SH2 domain of c-Src did not significantly contribute to the binding of tyrosine-phosphorylated proteins in v-*crk* c-*src* cells. v-Src formed complexes with other proteins, such as pp130 and pp110 (Fig. 7) (45).

DISCUSSION

The data presented here demonstrate that overexpression of c-Src and v-Crk can cause an elevation of tyrosine phosphorylation of cellular proteins and morphological transformation of rat 3Y1 fibroblast cells, whereas overexpression of v-Crk or c-Src alone causes only marginal transformation. We have also shown that the morphology of these v-*crk* c-*src* cells can revert to normal by Csk overexpression. Cellular tyrosine phosphorylation was also reduced to some extent after reversion by Csk overexpression. We have detected a good correlation between the degree of transformation or reversion and the levels of expression of

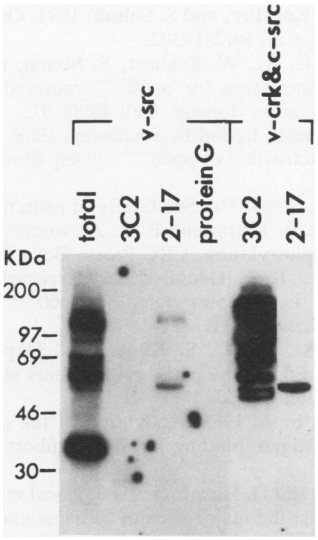

FIG. 7. Analysis of tyrosine-phosphorylated proteins coprecipitated with v-Crk or c-Src from v-*crk*/c-*src* transformed cells. Cell extracts prepared with 1% Nonidet P-40 buffer were incubated with either MAb 3C2 for v-Crk or MAb 2-17 for c-Src, and immune complexes were recovered with protein G-Sepharose as described in Materials and Methods. Immunoprecipitates were separated by SDS-PAGE and Western blotted with polyclonal antiphosphotyrosine antibodies. Total extract (50 μg) from v-*src* cells was used as a positive control (total), and protein G-Sepharose was used as a negative control (protein G). The same cell clones were used as described in the legend to Fig. 3.

v-Crk, c-Src, and Csk by analyzing the transfectants individually (data not shown). We also demonstrated that neither v-*crk*–c-*src*-transformed cells nor c-*src*527F-transformed cells could be reverted by Csk overexpression. v-Src is active in kinase activity as a result of truncation of the C-terminal region including Tyr-527 (17), and c-Src527F is kinase active as a result of mutation of Tyr-527 to Phe (22). We have also found that the overexpression of a Csk mutant inactive in kinase activity does not cause reversion in v-*crk* c-*src* cells (47). Moreover, overexpression of the SH2 domain of Csk alone is not sufficient to cause reversion (47). Thus, our data suggest that Csk acts on Tyr-527 of c-Src to suppress the morphological transformation of v-*crk* c-*src* cells. Moreover, in vitro kinase assays revealed that c-Src activity in v-*crk*–c-*src*-transformed cells was elevated three- to fourfold compared with that in c-Src-overexpressing cells and was restored to basal levels in *csk*-reverted cells. This is the first demonstration that Csk can suppress c-Src kinase activity in mammalian cells.

v-Crk causes elevation of the kinase activity of overexpressed c-Src without binding to c-Src. On the basis of the hypothesis that phosphorylated Tyr-527 may interact specifically with the catalytic domain or the SH2 domain of c-Src to suppress c-Src kinase activity (6, 9, 23, 27), it is conceivable that v-Crk activates c-Src by binding to the phosphorylated Tyr-527 and releasing the catalytic domain or the SH2 domain to interact with substrate proteins. However, we were unable to detect any significant formation of stable complexes of v-Crk and c-Src in vivo. Moreover, significant activation of c-Src kinase activity was not observed when the c-Src protein was incubated with v-Crk in vitro (data not shown). Therefore, activation of c-Src might be an indirect effect of v-Crk. Peptide mapping of in vivo phosphorylation sites revealed that c-Src proteins in both v-*crk*–c-*src*-transformed cells and *csk*-reverted cells were predominantly phosphorylated at Tyr-527 to a level similar to that in c-Src-overexpressing cells. As complete dephosphorylation of Tyr-527 in vitro increased c-Src kinase activity about 20-fold (11), a 10 to 20% decrease in Tyr-527 phosphorylation could account for the 3- or 4-fold increase in c-Src activity seen in v-*crk*–c-*src*-transformed cells. However, because 20 to 30% fluctuations in these kinds of experiments can occur, as described by Bagrodia et al. (2), small changes in phosphorylation of Tyr-527, sufficient to account for the increase in c-Src kinase activity in v-*crk* c-*src* cells, cannot be quantitated. If we assume that the phosphorylation of amino acids other than Tyr-527 or Tyr-416 does not affect c-Src kinase activity in vitro (9–11, 13, 17, 35, 51) and that Csk has a tight specificity for c-Src Tyr-527 in vivo (as shown here) or in vitro (37–39), a plausible explanation could be that v-Crk causes activation of overexpressed c-Src by reducing Tyr-527 phosphorylation of c-Src and that Csk overexpression restores Tyr-527 phosphorylation (see below).

***csk*-induced reversion and protein tyrosine phosphorylation.** *csk*-reverted cells still contain a higher level of tyrosine phosphorylation than that of v-*crk* cells and have a saturation density twofold higher than the type I v-*crk* cells or 3Y1 cells, even though the kinase activity of c-Src has been restored to near-basal levels. The cell clone used here was the flattest one we have ever obtained by Csk overexpression. This result may suggest that sustained tyrosine phosphorylation of these proteins does not require the activation of c-Src above basal levels (see below) or that another element, in addition to Csk, is necessary to downregulate c-Src kinase activity for certain substrates. It is also possible that much higher expression of Csk could suppress the increase in tyrosine phosphorylation. The level of v-Crk-induced phosphorylation of p130 is not changed after reversion, which suggests that p130 is phosphorylated by a kinase other than c-Src.

How does v-Crk contribute to the elevation of protein tyrosine phosphorylation, cellular transformation, and elevation of overexpressed c-Src kinase activity? Matsuda et al. (27) have shown that v-Crk can bind to a broad range of tyrosine-phosphorylated proteins: v-Crk binds to a variety of tyrosine-phosphorylated proteins found in v-*src*-, v-*yes*-, and v-*erb*B-transformed cells. It has been demonstrated in vitro that the binding of v-Crk to tyrosine-phosphorylated p130 protects v-Crk from dephosphorylation by a protein tyrosine phosphatase (3). Rotin et al. (46) reported a similar observation. They showed that the binding of the SH2 domain of phospholipase Cγ to tyrosine-phosphorylated epidermal growth factor receptor prevented receptor dephosphorylation. We observed that almost all tyrosine-phosphorylated proteins were bound to v-Crk in v-*crk* c-*src* cells (Fig. 7). Thus, it is likely that v-Crk contributes to the accumulation of tyrosine phosphorylation by binding to phosphorylated residues. This property of v-Crk might contribute greatly to cellular transformation. Indeed, it is interesting to note that 3Y1 (v-*crk*) clone 2 cells expressing high levels of v-Crk can be transformed by v-*src* cDNA with much higher efficiency than normal 3Y1 cells, or 3Y1(v-*crk*) clone 1 cells expressing much lower amounts of v-Crk (Table 1).

Overexpressed c-Src has been shown to be activated by an endogenous pathway at the G2-M transition of cell cycle (2, 8, 51). Therefore, substrate proteins may be phosphorylated by overexpressed c-Src activated transiently by such an endogenous pathway, and the swift binding of v-Crk to the

phosphorylated proteins could lead to their steady-state accumulation. A difference in timing of inactivation of the activated c-Src by Csk between v-*crk*–c-*src*-transformed cells and *csk*-reverted cells, may be due to a difference in the levels of Csk and may cause the differences of the levels and patterns of protein tyrosine phosphorylation between these two types of cells. In this context, Kanner et al. (19) reported that common tyrosine-phosphorylated proteins were detected in both Rous sarcoma virus- and CT10-transformed chicken embryo fibroblasts, although the phosphorylation levels were much lower in CT10-transformed cells than in Rous sarcoma virus-transformed cells. This may suggest that substrate proteins phosphorylated by endogenous c-Src kinase accumulate by the binding of v-Crk to the proteins in CT10-transformed chicken cells.

It remains to be explained why overexpressed c-Src is activated above basal levels in v-*crk* c-*src* cells. Even if the overexpressed c-Src is activated by the reduction of Tyr-527 phosphorylation as we discussed above, how v-Crk is involved in such an activation is still unclear. Three possible mechanisms of reducing Tyr-527 phosphorylation can be postulated: (i) inhibition of Csk kinase activity, (ii) activation of a protein tyrosine phosphatase specific for Tyr-527, and (iii) inhibition of a protein tyrosine phosphatase specific for Tyr-416, which would retain c-Src in its active state. Since these enzymes regulate tyrosine phosphorylation, it is plausible that their activities could be affected by irregular accumulation of tyrosine phosphorylation caused by v-Crk.

The system we described here could be used to analyze the mechanism of v-Crk transformation. We are currently analyzing the c-Src regulatory proteins. We have produced recombinant chicken Csk using the baculovirus system, and preliminary analysis showed that v-Crk did not affect Csk kinase activity in vitro (47).

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